ANTI-MALIGNANT PROPERTY OF CURCIN THROUGH INHIBITION OF TYROSINE KINASE AS EVIDENCED BY *IN-SILICO* STUDY

A Thesis Submitted In Partial Fulfillment

Of the requirement for the degree of

Bachelor of Technology

Biotechnology

By

K. Sandeep

107BT001

Department Of Biotechnology and Medical Engineering

National Institute of Technology, Rourkela

2011

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Under guidance of

Dr. B.P. Nayak

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2011

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Certificate

This is to certify that the work in the thesis entitled "***Anti-malignant property of Curcin through inhibition of tyrosine kinase as evidenced by in-silico study"*** by ***K. Sandeep*** in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology Engineering in the department of Biotechnology and Medical Engineering, National Institute of Technology, Rourkela is an authentic research work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the report has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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(K. Sandeep)

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List of Abbreviations

1. **RIP:** Ribosome Inactivating Protein
2. **EGFR:** Epidermal Growth Factor Receptor
3. **mTOR:** mammalian Target Of Rapamycin
4. **HIV:** Human Immunodeficiency Virus
5. **NCI:** National Cancer Institute
6. **rRNA:** ribosomal Ribo Nucleic Acid
7. **EGF:** Epidermal Growth Factor
8. **TGF:** Transforming Growth Factor
9. **MAPK:** Mitogen Activated Protein Kinase
10. **JNK:** c-Jun N terminal Kinases
11. **DNA:** Deoxy ribo Nucleic Acid
12. **PCI:** Potato Carboxypeptidase Inhibitor
13. **BLAST:** Basic Local Alignment Search Tool
14. **FORTRAN:** Formula Translation (High Level Language)
15. **NMR:** Nuclear Magnetic Resonance
16. **NCBI:** National Center for Biotechnology Information
17. **PDB:** Protein Data Bank
18. **MSA:** Multiple Sequence Alignment

Abstract

Among wide variety of plants only few species are able to produce milky fluid called latex. This contains a complex mixture of plant proteins like lectins, chitinases, proteinases etc. which exhibit a specific biochemical property. *Jatropha curcas*, a perennial plant widely found in tropical and sub tropical region is well known for its great capacity of producing latex. The plant mainly grown for biodiesel production has potential medical applications for a variety of human diseases. The latex of Jatropha contains alkaloids and lectins like Curcin, a protein which is proposed to exhibit antitumor properties. This protein has a sequence similarity with RIP family of proteins which are known for their anti cancerous nature. Preliminary studies suggested the use of Curcin as a part of antitumor treatment. Changing lifestyle and ageing in the developing world has resulted in rising number of individuals suffering from cancer. In order to assist further development in cancer related drug discovery, naturopathy and to widen the scope of research an *in-silico* study was performed in search for potential targets of Curcin which can be exploited to treat cancer. Homology modeling was done to predict the structure of Curcin molecule and protein-protein docking operation was carried out using EGFR and mTOR as target receptors. Interestingly the results went in the favor of Curcin efficiently proving its anti malignant property. However, the predicted model has to go for clinical trials to establish its effectiveness as an inhibitor of tumors caused by EGFR over expression.

Keywords: *Jatropha curcas,* anti malignant, Curcin, EGFR, *in-silico* study.

Chapter 1

Introduction

Introduction

Latex is widely distributed in plants and about 12,000–35,000 species have been reported to contain it. It is a milky fluid consisting of a liquid serum holding, either in suspension or solution, constituting a complex mixture of molecules. A wide range of proteins is found in latex fluids, such as carbohydrate-binding proteins (lectins) and *N*-acetyl-β-d-glucosaminidases which were purified from *Hevea brasiliensis* latex. Chitinases were detected in *Carica papaya* and *Ficus microcarpa* latexes. Proteolytic enzymes (serine and cysteine types) are more profuse proteinases found in latex fluids. Cysteine proteinases have been recently purified to homogeneity from distinct latex fluids and papain which is found in the latex of *C. papaya*, has been constantly studied since it was discovered. Evidences have supported the possible involvement of latex proteins in the plant defense mechanisms. However, additional information about its occurrence, biological activities and structure of latex proteins is still required to confirm this hypothesis.

Jatropha curcas (Euphorbiaceae) is a small shrub of about 5- 8 m in height. It has a soft bark and milky latex. It is a drought resistant, perennial plant that grows even in the marginal and poor soil.

Figure 1.1: Image of *Jatropha curcas* plant

The *Jatropha curcas* is found mainly in tropical regions and to a lesser extent in sub-tropical areas. The plant is well-known for its great capacity of producing latex which exudates from the stem. There is an emergent demand in popular medicine for the different parts of the plant, including the latex, and as a

consequence a vast literature describes it's the related potential. However there is inadequate information about biochemical properties of the latex of *Jatropha curcas*.

The latex is used for treating tumors; ulcers and wound healing in traditional medicine, the leaves of the plant are used as a haemostatic and are also drastic purgative. The major constituents of latex are lectins and phorbolesters, curcacycline-A, jatrophine and curcin. These constituents indicate pharmacological properties like coagulant activity of the latex from *Jatropha curcas* showed that whole latex significantly reduced the clotting time of human blood. Diluted latex, though, prolonged the clotting time: at high dilutions, the blood did not clot at all. This shows that this latex possesses both procoagulant and anticoagulant activities.

The motivation factor of this study is the lack of information available about latex proteins from *Jatropha curcas*. Some preliminary experiments which were conducted earlier in our laboratory suggested that the latex could be used as a part in antitumor treatment and also treatment of various other diseases. In an attempt to investigate this hypothesis, further *in-silico* characterization of the latex protein was performed to obtain new information on the occurrence and its biological activity.

Chapter 2

Plants of pharmacological importance

Plants of pharmacological importance

Various ethno-pharmacological surveys conducted among herbal practitioners have revealed a large number of laticiferous plant species are used as source of herbal therapies. Plant derived compounds have played an important role in the development of several anticancer agents. Almost all present medicines come from medicinal plants and are obtained from research on medicinal plants. Ethno medicines were earlier used by hunters for themselves and their hunting dogs. Plant based medicines are used to treat snakebites, scorpion stings, for injuries and to help hunting success. With the altering pattern of life style most of the diseases are now becoming common. The traditional system of medicine based on ancient knowledge are chiefly concerned with building the body strength which can assist in healing the ailments and these systems rely largely on the nature cure (Kumar *et al.,* 1995). The Ayurvedic system has described a large number of such medicines based on plants or plant product and the determination of their morphological and pharmacological or pharmacognostical characters can give a better understanding of their active principles and mode of action. Many plants contain proteins that are capable of inactivating ribosome and accordingly are called ribosome-inactivating protein (RIP) (Barbieri *et al*., 1993).

Modern investigations point out that extracts of *Aloe vera* act on the dead epithelial cells of the skin, aiding their removal from the surface and stimulating the growth of new cells. Thus Aloe is a great gift of traditional medicine for shielding the smooth skin of human beings especially when radiation damage has assumed an alarming situation due to stratospheric ozone depletion. Fresh juice of leaves are also used in liver and spleen troubles and also for eye troubles, found helpful in X-ray burns, dermatitis, coetaneous and other skin disorders (Choudhary *et al.,* 2001). *Calotropis procera* is one of the important numbers of traditional herbal medicinal plant in every home of

India. The leaves of *C. procera* are warmed and tied around any body part which is in pain. It is practically helpful in backache and in joint pains. Leaf latex if applied on fresh cut it stops bleeding without delay (Hattori *et al.,* 1993).

Jatropha curcas: Its juice is a well known purgative and is helpful in whitlow, convulsions, syphilis, neuralgia, dropsy, anasarca, pleurisy and pneumonia. Root bark is applied on the outside in rheumatism and is used in sores. It is helpful in chronic dysentery, thirst, abdominal complaints, biliousness, anemia, fistula, ulcer, and diseases of the heart and skin (Kumar *et al.,* 2000).

Euphorbia hirta is demulcent, antispasmodic, antiasthmatic pectoral, anthelmintic and local parasiticide. Plant is mainly used in the affections of childhood, in worms, bowel complaints and cough, in postnatal complaints, failure of lactation, breast pain. Latex is vermifuge and applied in diseases of urino-genitory tract and also in application for warts. *Euphorbia tirucalli* is useful in biliousness, leucorrhoea, leprosy, dropsy, whooping asthma, enlargement of spleen, dyspepsia, jaundice, colic tumors, and stones in bladder. Milky juice is useful to itch and scorpion bites (Sharma *et al.,* 2001).

Of the tested extracts, the methanol extracts of the plants *Acacia nilotica* (bark), *Euphorbia granulata* (leaves), *Maytenus senegalensis* (stem-bark) and aqueous extracts of *A. nilotica* and *M. senegalensis* showed significant inhibitory effects against HIV-1 PR (Hussein *et al.,* 1999). *Ganoderma lucidum* has been described to have triterpenes which have inhibitory effects against HIV-1 protease (Min *et al.,* 1998).

Euphorbiaceae is a large family of about 300 genera and 6000 or more species. Most members are trees or shrubs and few are herbs. Some genera (e.g. *Euphorbia*) are xerophytic (Evans *et al.,*

2002). The family of Euphorbiaceae contains many members from milkweeds to poinsettias and covering ground from the Americas to India. Euphorbiaceae members have been used in treating gonorrhea, asthma, spleen enlargement, as well as tumors (Tiwari *et al.*, 2005). By using a small amount of the milky latex or parts of the bark and stem, it does produce a medicinal effect but should be noted that in highly concentrated doses has adverse effects. The latex of *Euphorbia tirucalli* has also been researched to eliminate the presence of fascioliasis (Tiwari *et al.,* 2004). *Euphorbia* species have been reported to possess antitumor activity and was recommended to be used as anticancer remedies; on the other hand some *Euphorbia* species have been reported as carcinogens. Several other species have been reported to exhibit antimicrobial, antimalarial, insecticidal, mollusicidal, anti-inflammatory and antipyretic activities (Natarajan *et al.,* 2005).

Chapter 3

Jatropha curcas

(दन्ती,प्रत्यक्श्रेणी)

Jatropha curcas *(दन्ती, प्रत्यक्श्रेणी)*

Jatropha curcas, a multipurpose, drought resistant, perennial plant belonging to Euphorbiaceae family is achieving a lot of importance for the production of biodiesel. It is a tropical plant that can be grown in low to high rainfall areas either in the farms as a commercial crop or on the boundaries as a hedge to defend fields from grazing animals and to prevent erosion (Kumar *et al,*.2008).

The genus *Jatropha* belongs to tribe Joannesieae in the Euphorbiaceae family and consists of approximately 170 known species. Linnaeus (1753) was the first to name this physic nut *Jatropha* L. in "Species Plantarum" and this is still applicable today. The genus *Jatropha* contains approximately 170 known species (Agaceta *et al.,* 1981). The genus name *Jatropha* derives from the Greek *jatros* (doctor), *trophe* (food), which implies its medicinal uses (Felke *et al*., 1914). The lifetime of the Jatropha curcas plant is more than 50 years. The physic nut, by definition, is a small tree or large shrub, which can reach a height of three to five meters, but under favourable conditions it can attain a height of 8 or 10m. The plant displays articulated growth, with amorphological discontinuity at each growth. The branches have latex. Normally, five roots are produced from seedlings, one central and four peripheral. A tap root is not generally formed by vegetatively propagated plants. Leaves five to seven lobed, hypostomatic and stomata are of paracytic (Rubiaceous) kind.

The species, though native to America, is currently almost pantropical and widely planted as a medicinal plant which soon tends to establish itself. It is listed as a weed in Brazil, Fiji, Honduras, India, Jamaica, Panama, Puerto Rico, and Salvador (Holm *et al.*, 1979).

A diversity of plants contains endopeptidases in their latex as part of their protein constituents. For example, latex from the unripe fruit of the papaya *Carica papaya* L. consists a mixture of cysteine endopeptidases, such as papain (EC 3.4.22.2), chymopapains A and B (3.4.22.6), papaya endopeptidase III, papaya endopeptidase IV (1, 2) and a just identified one designated as endopeptidase W. Fresh or dried latex obtained from stem or seeds of *J.curcas* following injury contains proteolytic enzymes. The bleeding procedure goes on for a few minutes until a latex clot forms on the affected area. This process of defense resembles blood coagulation and clot formation during wounding in mammals (Silva *et al.,* 1997).

3.1 Possible uses of *Jatropha curcas* plant

Jatropha has become famous mainly for the production of biodiesel; besides this it has several medicinal applications too. Most parts of this plant are used for the treatment of various human and veterinary ailments (Shukla *et al.,* 1982). The white latex serves as a disinfectant in oral cavity infections in children. The latex of Jatropha contains alkaloids including Jatrophine, Jatropham and curcain with anti-cancerous properties (Montanaro *et al.,* 1973). It is also used superficially against skin diseases, piles and sores among the domestic livestock.

All parts of Jatropha (seeds, leaves and bark) have been used extensively in traditional medicine and for veterinary purposes for a long time. Some compounds (Curcin, Curcacycline A) with antitumor activities were reportedly found in this plant (Berg *et al.*, 1995). Substances such as phorbol esters, which are poisonous to animals and humans, have been isolated and their molluscicidal, insecticidal and fungicidal properties have been confirmed in lab-scale experiments and field trials (Nwosu and Okafor, 1995).

Table 3.1 : Chemical composition of its various parts of *J.curcas*		
Various parts	**Chemical composition**	**References**
Aerial parts	Organic acids (*o* and *p*-coumaric acid, *p*-OH-benzoic acid, Protocatechuic acid, Resorsilic acid) Saponins and Tannins	Hemalatha and Radhakrishnaiah (1993)
Stembark	β- Amyrin, β- Sitosterol and Taraxerol	Mitra et al. (1970)
Leaves	Cyclic triterpenes stigmasterol, stigmast-5-en-3β, 7α -diol, stigmast-5-en-3 β, 7 β -diol, cholest-5-en-3 β,7α-diol, cholest-5-en-3fl,7 β -diol, campesterol, sitosterol, 7-keto- β sitosterol as well as the -d-glucoside of sitosterol. Flavonoids apigenin, vitexin, isovitexin. Leaves also contain the dimer of a triterpene alcohol ($C_{63}H_{117}O_9$) and two flavonoidal glycosides.	Khafagy et al. (1977) Mitra et al. (1970), Khafagy et al. (1977), Hufford and Oguntimein (1987)
Latex	Curcin, Curcacycline A, a cyclic octapeptide	Nath and Dutta (1991) Van den Berg et al. (1995)
Seeds	Curcin, a lectin Phorbolesters Esterases Lipase	Staubmann et al. (1999) Stirpe et al. (1976) Adolf et al. (1984), Makkar et al. (1997)
Kernel and press cake	Phytates, Saponins Trypsin inhibitor	Aregheore et al. (1997), Makkar and Becker (1997), Wink et al. (1997)
Roots	β Sitosterol and its β -d-glucoside, marmesin, propacin, the curculathyranes A and B and the curcusones A-D. Diterpenoids jatrophol and jatropholone A and B, the coumarin tomentin, the coumarino-lignan jatrophin as well as taraxerol.	Naengchomnong et al. (1986, 1994)

The leaves have apigenin, vitexin and isovitexin etc. which along with other factors enable them to be used against malaria, rheumatic and muscular pains (Duke *et al*., 1981). Antibiotic activity of Jatropha has been observed against organisms including *Staphylococcus aureus* and *Escherichia coli* (Thomas *et al*., 2003). There are some chemical compounds counting curcin (an alkaloid) in its seeds that make it unsuitable for common human consumption. The roots are well-known to contain an antidote against snake venom. The root extract is also used to check bleeding from gums. The leaf, fruits, latex and bark contain glycosides, tannins, phytosterols, flavonoids and steroidal sapogenins that exhibit broad ranging medicinal properties. The plant products exhibit anti-bacterial and anti-fungal activities (Debnath *et al.,* 2008). The latex from *Jatropha curcas* promotes healing of wounds, refractory ulcers, septic gums and in cuts and bruises. The wound healing property was observed due to the presence of curcin, the protease isolated from the latex from the plant *J. curcas*. Healing of the wound by the curcin ointments was found to be better than nitrofurazone ointment and propamidine isethionate cream in mice (Nath *et al*., 1992).

Dried latex and chloroform extract of roots has been reported to consist of anti-inflammatory activity (Basu *et al*., 1994). Aqueous extract of the latex has been found to exhibit analgesic, antipyretic and anti-inflammatory activity. A single dose of the aqueous suspension of the dried latex was effective to a significant level against the acute inflammatory response (Kumar *et al*., 1994).

The latex of *Jatropha curcas* has also been used for the synthesis of silver nanoparticles. Some of the major components in the latex of *Jatropha curcas* were identified as curcin, curacycline A and curacycline B. The silver nanoparticles obtained using this source had two broad distributions- one having particles in the range of 20-40 nm and the other having larger and uneven particles.

Molecular modeling studies of the peptides in the latex revealed that the silver ions were first entrapped in the core structure of the cyclic structure of the protein and were then reduced and stabilized *in situ* by the amide group of the peptide. This resulted in particles with radius similar to the peptides. It was also found that the larger particles with uneven shapes were stabilized by the enzyme curcain (Bar *et al*., 2009).

Many of these traditional medicinal properties of *Jatropha curcas* are required to be investigated in depth for the marketable therapeutic products along with the toxicological effects thereof.

3.2 Pharmacological properties of latex

Plant latex is the cytoplasm of extremely specialized cells known as laticifers. Proteins are abundantly accumulated in latex and are involved in the defense system. One important characteristic of laticifers is that they have various toxic compounds in the latex; for example, the neurotransmitter dopamine in the Persian poppy (*Papaver bracteatum*), narcotic alkaloid morphine in the opium poppy (*Papaver somniferum*), and insecticidal compounds such as the glycosidase inhibitors 1,4-dideoxy-1,4-imino-d-arabinitol (d-AB1) and 1-deoxynojirimycin (DNJ) in mulberry In addition, cysteine protease in latex of papaya (*Carica papaya*) and wild fig (*Ficus virgatalatex*) is lethal to caterpillars of herbivorous insects. The latex itself is a secondary metabolite and is combination of compounds such as phytosterols, amine oxidases and as well as many others (Phelps *et al*., 2008). The latex itself has been found to be powerful inhibitors to watermelon mosaic virus (Tewari and Shukla, 1982). The leaves and latex are used in healing of wounds, refractory ulcers, and septic gums and cuts and bruises. A proteolytic enzyme (curcain) has been

reported to contain wound healing activity in mice (Nath and Dutta, 1997; Villegas *et al.,* 1997). Investigation of the coagulant activity of the latex of *Jatropha* showed that whole latex notably reduced the clotting time of human blood.

Curcin, a protein obtained from seeds and latex of Jatropha curcas is proposed to have antitumor properties (Lin Juan *et al.,* 2003). This is one of the major proteins in the *Jatropha curcas* latex, is similar to Ricin & Abrin proteins of *Ricinus communis* and *Abrus precatorius,* respectively in function like in inhibiting protein synthesis in whole cells and in cell-free systems**.** (Baloch *et al.,* 2005) found such properties are contained by the latex that supports this. But the elaborate studies regarding the structural and functional properties of curcin and the mechanism of action has not been reported. The anticancerous property of curcin on gastric cancer cell line and human hepatoma has proved (Juan *et al*., 2003).

Different effects of curcin on different cells examined were observed, with SGC-7901, Sp2/0 and human hepatoma being the most sensitive to curcin, and HeLa cells being the most resistant to curcin. The curcin had a powerful inhibitory action upon protein synthesis in reticulocyte lysate with an IC_{50} (95% confidence limits) value of 0.19 (0.12-0.27) nmol/L. The IC_{50} (95% confidence limits) of curcin on SGC-7901, Sp2/0, and human hepatoma was 0.23 (0.16-0.32) mg/L, 0.66 (0.36-0.97) mg/L, 3.16 (2.75-3.58) mg/L, respectively. Curcin was found to have no toxic to HeLa cells and normal cells (MRC). This shows that curcin is suitable for the preparation of immunotoxins (L. Williams and D.J. Newman, 2005).

The evaluation of anticancer and anti-HIV activities of latex extracts from nine medicinal plants collected in Zaire was carried out against the National Cancer Institute (NCI) panel of human tumor cell lines provided for the preliminary screening of natural products. The methanol extracts from the root bark of *Hymenocardia acida*, the stem bark of *Mangifera indica* and the leaves of *Sida rhombifolia*, exhibited cytotoxic activity against the 60 human cell lines was checked. Out of these, the methanol extract from *M. indica* produced a remarkable interesting pattern of differential cellular sensitivity against the MDA-MB-231, MDA-MB-435, and MDA-N human breast cancer cell lines. In the AIDS-antiviral screen, the methanol extract from *Jatropha curcas* was found to produce a moderate cytoprotective effect against HIV in cultured human lymphoblastoid CEM-SS cells (Muanza *et al., 1995).*

3.3 Antitumor action mechanism of latex

Curcin a major constituent of *J. curcas* latex, is frequently classified as a lectin and described as being comparable to ricin from castor bean with the implication that it has similar toxicity. But all of these are incorrect statements. Both curcin and ricin are ribosome inactivating proteins (RIPs), which depurinate rRNA, thus inhibiting protein synthesis (Andrew *et al.,* 2009). Thus curcin is classified under the RIP superfamily of proteins.

For a long time the attention in RIP (ribosome-inactivating proteins) has been focused on developing antitumor drugs that selectively target to tumor cells. A comparison of the amino acid sequences of curcin with other RIP, e.g., ricin A-chain and trichosanthin revealed that there existed relatively high resemblance among them. The percentages of identity between curcin and ricin A-

chain, and between curcin and trichosanthin were found to be 54 % (156/287), 57 % (138/241) respectively (Chow *et al.,* 1990).

The RIPs are RNA N-glycosidases (Barbieri *et al.,* 1993) that inactivate ribosomes by site-specifically cleaving the single N–C glycosidic bond between adenine and ribose at A4324 in the 28s rRNA. The depurination of the specific adenine prevents the elongation factor (EF-2) from binding to the 60S subunit, thus RIPs can inhibit protein synthesis. Interest in RIPs has arisen from their potential medical and therapeutic applications because many of these proteins have been found to be more toxic to tumor cells than normal cells (Lin et al., 1970). Studies have shown that they can be used as toxic components of immunotoxins targeted at tumor cells (Bolognesi *et. al.,* 2004), abortifacients (Jin *et. al.,* 1985) and antiviral agent (Au et al., 2000; Wang et al., 2002) in patients suffering with AIDS.

On the basis of structure of the genes and mature proteins (Van Damme *et al.,* 2001), RIPs are classified into three types. Type 1, such as trichosanthin (Pan *et al*., 1993), bryodin (Stirpe *et al.,* 1986), a,b-momorcharin (Husain *et al.,* 1994; Yuan *et al.,* 1999) and luffin a, b (Kamenosono *et al.,* 1988), consist a single polypeptide chain and have alkaline isoelectric points. They have potent capabilities to inhibit protein synthesis in the cell free system, but are relative non-toxic to the intact cell. Type 2, such as ricin (Rutenber *et al.,* 1991) and abrin (Tahirov *et al.,* 1995), having two chains, chain A and chain B, linked by disulfide bridges. The A chain have the ribosome-inactivating property; the B chain contains a lectin domain which interacts with the cell surface galactosides and facilitates the entry of the A chain into the cytoplasm of the cell. Thus, some, but

not all, type 2 RIPs have stronger toxicities than type 1 RIPs because type 1 RIPs can only enter into cells with difficulty, even though they are very active towards isolated ribosome.

Type 3 is one kind of jasmonate-induced protein, such as JIP60 (Chaudhry *et al.,* 1994) from maize. It consists of an N-terminal domain similar to other type 1 RIPs and dissimilar C-terminal domain of unknown function.

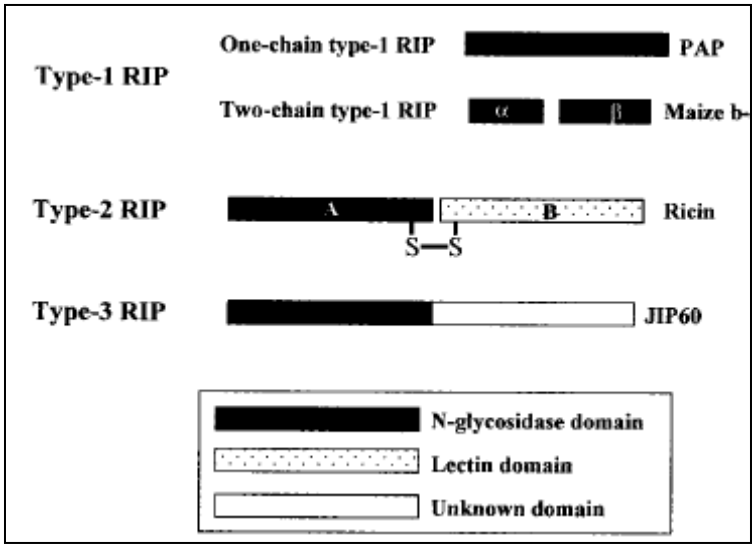


Figure 3.1: Structure of Different Types of RIPs

Curcin, a protein isolated from latex of *J.curcas* shows sequence similarity in amino acid with RIPs (Chow *et al.,* 1990). Significantly, it can inhibit the proliferation of tumor cells, through the induction of apoptosis and differentiation (Xie *et al.,* 2006) of cancer cells. In this study, we describe the structural and functional analysis of curcin, from the latex of *J.curcas*. The objective would be to find an evidence of anti-malignancy of the latex protein by *in-silico* approach.

Chapter 4

Targeted pathways of cancer

Targeted Pathways of Cancer

The latex protein, Curcin has already been proved to act by inhibiting protein synthesis, the characteristic of all RIP family of proteins. Apart from this there are other major pathways which contribute to large number of cancerous ailments. The most common being the cancer caused by epidermal growth factor receptor over expression.

The Epidermal Growth Factor Receptor is the cell surface receptor member of EGF family protein ligands. It consists of four closely related tyrosine kinase receptors: EGFR (ErbB1), HER2/c-neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). The receptor is present on the cell surface and is activated by specific ligands like EGF and TGF-alpha. It functions by homo-dimerization of hetero-dimerization with other family member which in turn activates its intrinsic intracellular protein-tyrosine kinase activity. The autophosphorylation of several tyrosine residues in EGFR activates signaling by several downstream proteins in signal transduction cascades, like MAPK, AKT and JNK pathways. This leads to DNA synthesis, cell migration, adhesion and proliferation. Any mutation that leads EGFR over expression or up regulation is associated with a number of cancers, including lung, anal and glioblastoma

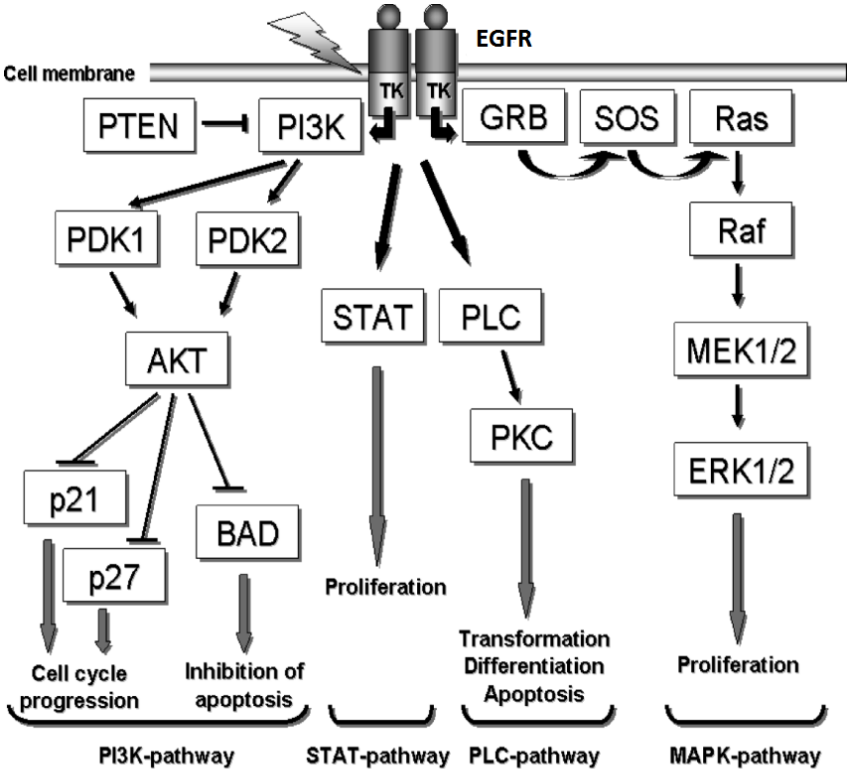


Figure 4.1 : Various EGFR signaling pathways

multiforme. This mutation leads to its constant activation resulting in uncontrolled cell division. This phenomenon is thus a target of many anticancer therapeutic approaches like cetuximab, erlotinib which are either monoclonal antibody inhibitors or small molecule kinase inhibitor.

Some naturally occurring inhibitors like Potato Carboxypeptidase Inhibitor (PCI) contains a small cysteine module, called a T-knot scaffold, which is also found in some EGF family proteins shows antagonistic properties due to the structural similarities. The potato protease inhibitor peptide forms complexes with several metallo-carboxypeptidases and inhibits them in a competitive way. It consists of 39 amino acids forming a globular core stabilized by three disulphide bridges and a C-terminal tail. It inhibits receptor dimerization and receptor trans-autophosphorylation by EGF. PCI blocks the formation and activation of ErbB1/HER2 heterodimers which plays a prominent role in carcinoma development. It also inhibits Transforming Growth Factor alpha (TGF-a) which is up regulated in some human cancers.

Apart from this other cancer related pathways like that involving Mammalian Target of Rapamycin (mTOR) was also taken into consideration in the present study to widen the span of research. The mTOR is a serine/threonine protein kinase that regulates cell growth, survival, proliferation, motility, protein synthesis and transcription. Its inhibition leads to apoptosis or arrest of cells in G1 phase of cell cycle.

Chapter 5

Methodology

Methodology

5.1 Tools used to perform *in-silico* study

5.1.1 Sequence Alignment Tools

Sequence alignment is a powerful way to compare novel sequences with previously characterized genes. Functional, structural and evolutionary information can be inferred from fine designed queries and alignments.

BLAST

The NCBI defines Basic Local Alignment Search Tool (BLAST) as a tool that finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. As the BLAST algorithm detects local as well as global alignments, regions of similarity embedded in otherwise unrelated proteins can be detected. Both types of similarity may provide significant clues to the function of uncharacterized proteins. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

CLUSTAL X

Clustal X offers a graphical interface for the Clustal W multiple sequence alignment programs. It provides an integrated situation for performing multiple sequence and profile alignments and analyzing the results. The output of the sequence alignment is then displayed in a window on the screen. Progressive MSA helps to create a guide and to identify the consensus sequence using Clustal X.

5.1.2 Structure Prediction Tools

The main objective of structural bioinformatics is the prediction of the 3D structure of a protein from its 1D protein sequence. The goal is to determine the shape or fold that a given amino acid sequence will adopt. If the two sequences show evolutionary ancestry, they are said to be homologous. For such similar sequence pairs we can create a structure of the query protein by choosing the structure of the known homologous sequence as a template. This is known as comparative modeling. The heart of the procedure is the selection of a suitable structural template based on sequence pair similarity. This is further extended by the alignment of query sequence to the template structure selected to build the backbone of the query protein. Finally the entire structure modeled refinement is done by loop construction and side-chain modeling. Focusing in the needs to solve the problem several modeling softwares or programs have been developed.

MODELLER 9v4

It is a program used for homology or comparative modeling of protein three-dimensional structures. Built on FORTRAN, it runs on python script file commands and is often used for homology or comparative protein structure modeling. MODELLER helps determine the spatial restraints from the templates. It generates a number of 3D models of the sequence you submit satisfying the template restraints. MODELLER automatically calculates a full-atom model protein 3D structure keeping in the constraints of spatial restraints. The restraints can be derived from a number of different sources like NMR experiments, cross-linking experiments, fluorescence spectroscopy, rules of secondary structure packing (combinatorial modeling), image reconstruction in electron microscopy, homologous structures (comparative modeling), site-directed mutagenesis, residue-residue and atom-atom potentials of mean force, etc. It is not

an automated tool instead is a very precise program. Any error in the format of the sequence alignment prevents the MODELLER from performing homology modeling. The program is very specific about the extension names of the file formats used for homology modeling. It is a very reliable program and it allows the user to specify what he wants in the end result. To choose the best possible structure from a collection of models, the program uses a standard DOPE energy function. The model with minimum molpdf value is chosen as the best probable structure.

SWISS MODEL

It is a fully automated protein structure homology modeling server, accessible via the ExPaSy web server or from a standalone program DeepView (Swiss Pdb-Viewer). The purpose of this server is to make Protein Modeling accessible to all biochemists and molecular biologists worldwide. It is developed by the Computational Structural Biology Group at the Swiss Institute of Bioinformatics (SIB) and the Biozentrum of the University of Basel. For a given target protein, a library of experimental protein structures is searched to identify suitable templates. On the basis of a sequence alignment between the target protein and the template structure, a three-dimensional model for the target protein is generated. Model quality assessment tools are used to estimate the reliability of the resulting models. The QMEAN4 score is a composite score consisting of a linear combination of 4 statistical potential terms (estimated model reliability between 0-1). The pseudo-energies of the contributing are calculated together with their Z-scores with respect to scores obtained for high-resolution experimental structures of similar size solved by X-ray crystallography and can be taken analogous to DOPE score method.

The present study is based on homology modeling using this web based modeling expert system.

5.1.3 Visualization Tools

UCSF Chimera (Chimera)

Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics (RBVI) at the University of California, San Francisco, a program for interactive visualization and analysis of molecular structures. It helps us to obtain the structure either directly from the PDB site or through many other formats, to localize the ligand that is in the active site and display the structure which is protein with the inhibitor. Secondary structure identification can be done, as it can show and hide ribbons. The secondary structure can observe motifs of the protein and also create a molecular surface around the protein, then color the surface according to different properties of the amino acids. Studying the protein-ligand interaction can be done by identifying the residues within 5.0A, and then identify the residues involved in the binding and label the residue names and types. Chimera shows the hydrogen bonds interaction between proteins and ligand.

Energy minimization

Before energy calculations can be performed, it is necessary to correct structural inconsistencies, add hydrogen, and associate atoms with force field parameters. Clicking Minimize dismisses the dialog (unless the option to Keep dialog up after Minimize is checked) and may call Dock Prep to perform several tasks to prepare the structure(s). Dock Prep may in turn call Add H and Add Charge. Each of the tasks is a checkbox option that can be turned off independently if already done or deemed unnecessary. Minimization routines are performed by MMTK, which is included with Chimera.

PyMOL (Link : www.pymol.org)

PyMOL is an open-source, user-sponsored, molecular visualization system made by Warren Lyford DeLano and commercialized by DeLano Scientific LLC, a private software company dedicated to creating useful tools that are universally accessible to scientific and educational communities. It is well established for producing high quality 3D images of small molecules and biological macromolecules such as proteins. It is one of few open source visualization tools available for use in structural biology. The Py portion of the software's name refers to the fact that it is extensible by the Python programming language. PyMOL is good for:

1. Viewing 3D Molecular Structures
2. Rendering Figures Artistically
3. Animating Molecules Dynamically
4. Giving Live 3D Presentations
5. Sharing Interactive Visualizations

5.1.4 Structure Validation tools

RAMPAGE (Link: http://mordred.bioc.cam.ac.uk/~rapper/rampage.php)

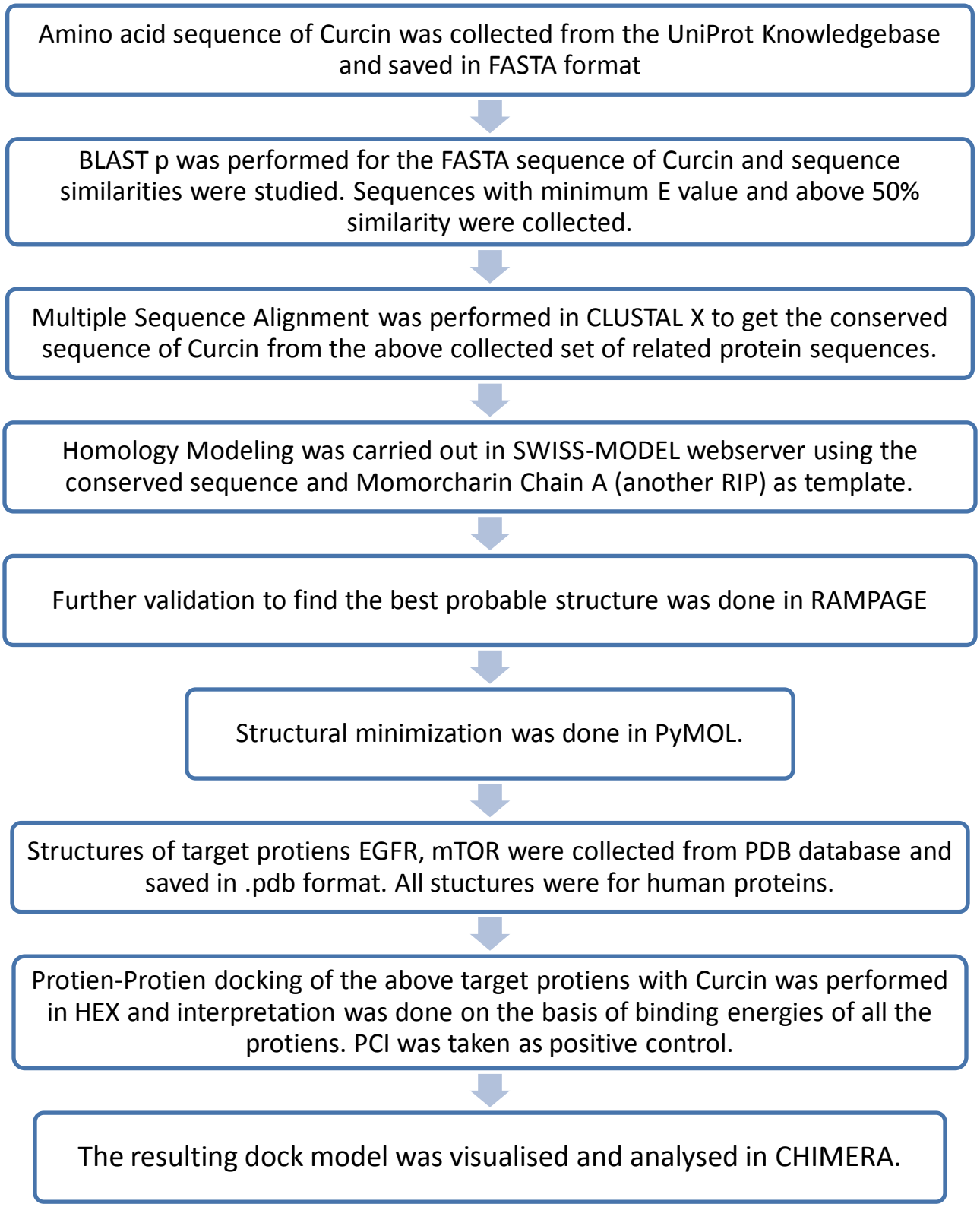
RAMPAGE is an online tool available for the user free of cost. This software is generally used for the secondary structure prediction of the proteins. It is based on Ramachandran Plot assessment of a protein model.

5.1.5 Docking Tools

HEX 6.3 and HEX_SERVER: Online server **(**Link: http://www.csd.abdn.ac.uk/hex_server)

HEX is good & trusted software for docking and free for academic usage. *HEX* recognizes protein and DNA structures in PDB format. It is an interactive protein **docking** and molecular **superposition** program. The aim of molecular docking is to accomplish an optimized conformation for both the protein and ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized. To carry out a docking screen, the first prerequisite is a structure of the protein of interest. This protein structure and a database of potential ligands provide inputs to a docking program. The accomplishment of a docking program depends on two components: the search algorithm and the scoring function. This is a free service without login or registration requirement.

5.2 Procedure followed for the *in-silico study*



Flowchart 5.1: Experimental plan for *in-silico* studies of Curcin

5.2.1 Sequence Alignment

First of all amino acid sequence was searched in NCBI for Curcin and saved in the FASTA format. After that using BLAST p in PDB server homologous sequence for Curcin with more than 50% sequence similarity were selected and their sequences and PDB structures were saved in FASTA format. When performing a BLAST search, a reliable first approach is to identify hits with a suitably low *E*-value, which are considered sufficiently close in evolution to make a reliable homology model.

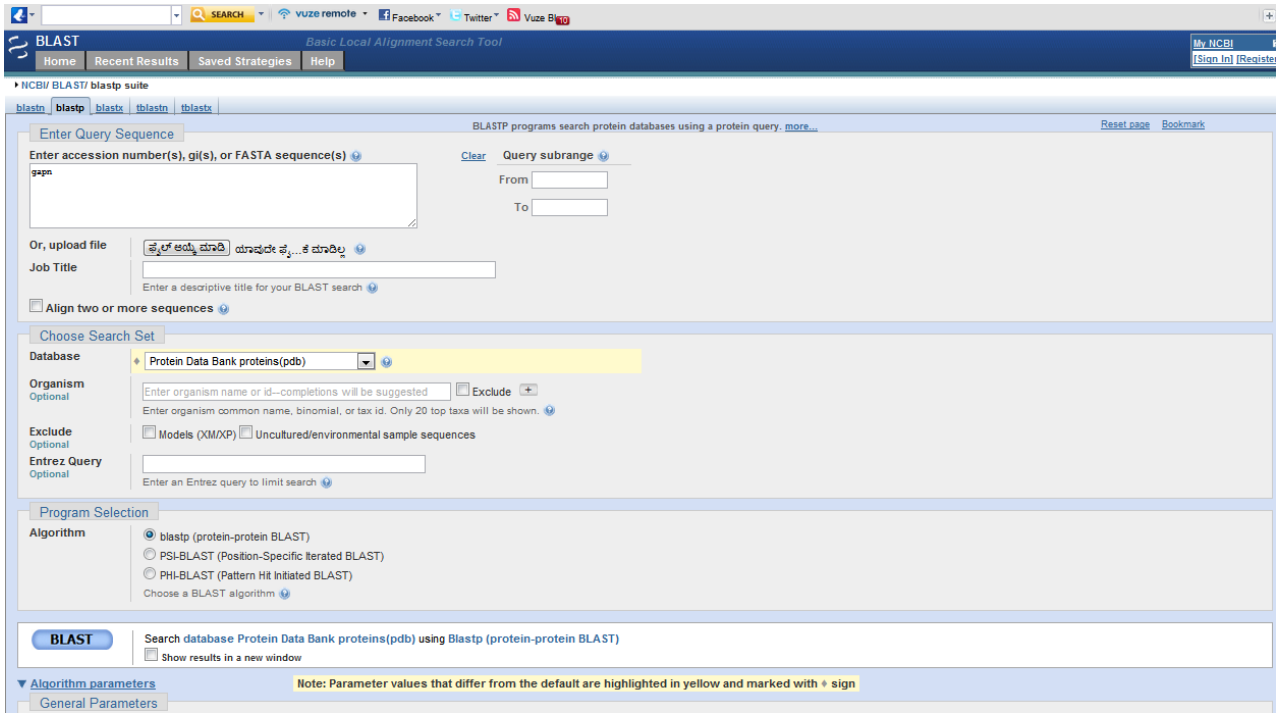


Figure 5.1: Screenshot of BLASTp window of NCBI

Further conserved residues were studied by using CLUSTAL X. The reference sequence input being that of Curcin which is compared with a set of sequence data generated from BLASTp earlier. The MSA data generated is included in the Appendix.

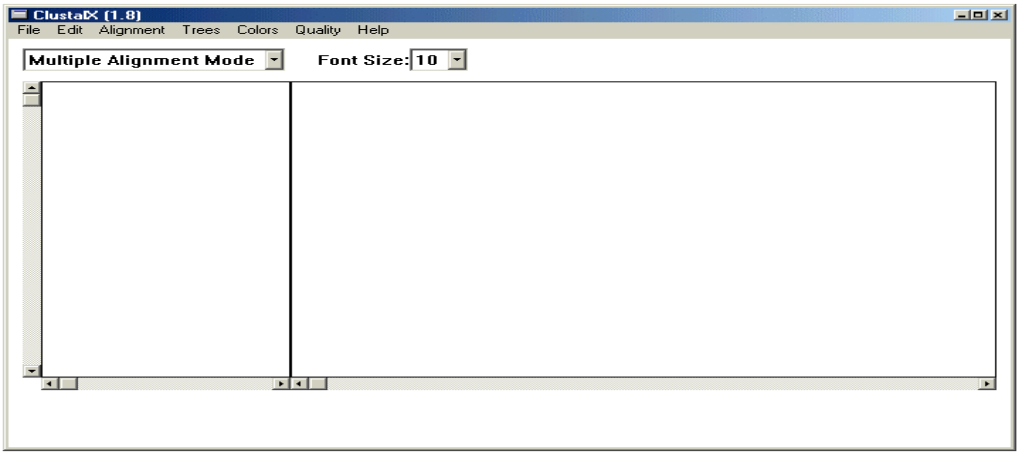


Figure 5.2: Screenshot of CLUSTAL X dialogue box

5.2.2 Homology Modeling and validation

Homology modeling is based on the logical assumption that two homologous proteins will share very similar structures. Because a protein's fold is more evolutionarily conserved than its amino acid sequence, a target sequence can be modeled with practical accuracy on a very distantly related template, provided that the relationship between target and template can be discerned through sequence alignment. Homology modeling is most accurate when the target and template both have similar sequences. Comparative ("homology") modeling approximates the 3D structure of a target protein for which only the sequence is accessible, provided an empirical 3D "template" structure is available with >30% sequence identity.

All homology models were constructed with the SWISS-MODEL web server. The best model on the basis of QMEAN4 score and Z- score was then saved in the PDB format. Further validation of the model was done by the RAMPAGE server which uses the PDB format as input.

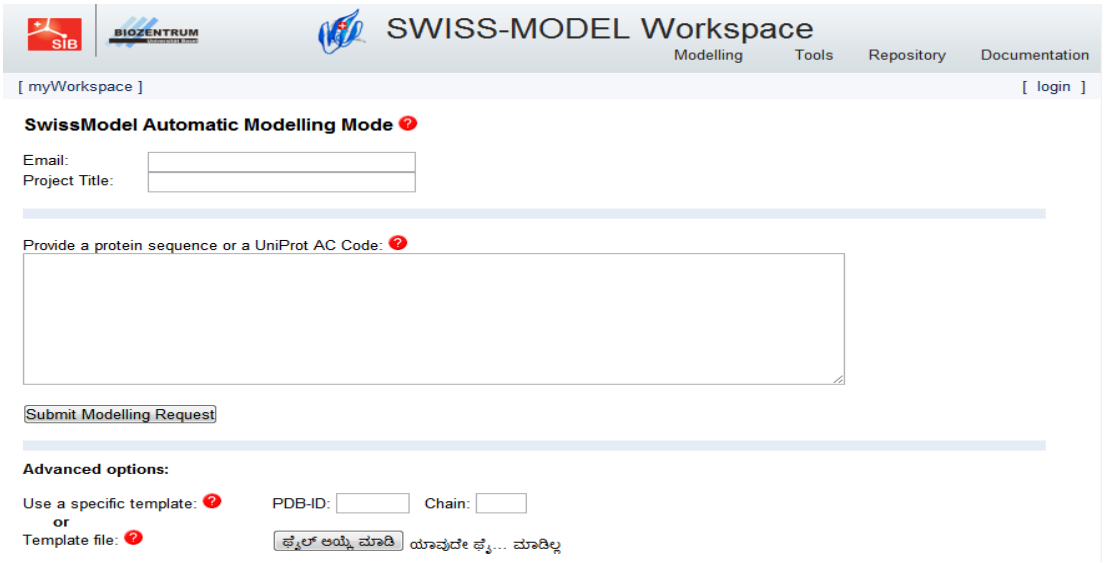


Figure 5.3: Web screen shot of SWISS MODEL server

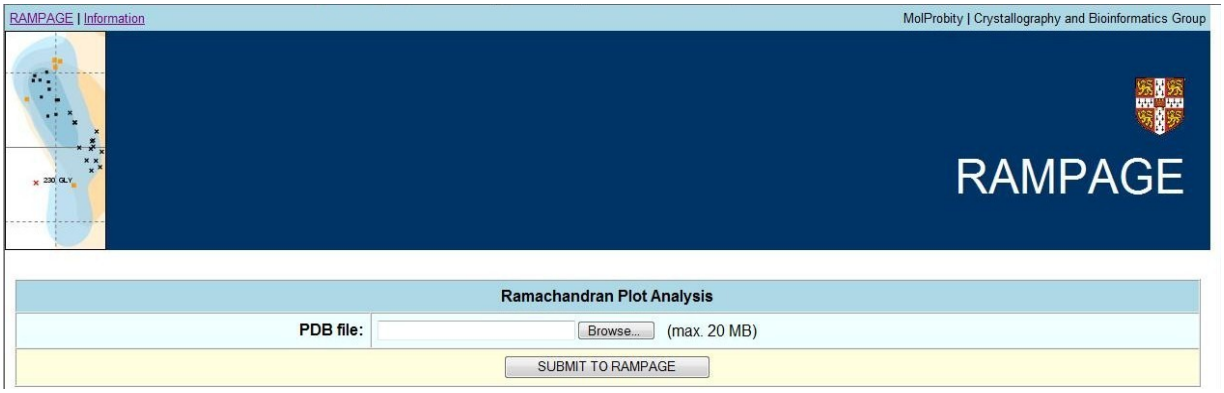


Figure 5.4: RAMPAGE server screenshot

5.2.3 Protein- Protein Docking

The validated model is used to study the receptor-ligand interaction using protein docking in HEX. The input for the docking program is given using the 'File > Open > Ligand' and 'File > Open > Receptor' option in the main window. The Docking and Orientation controls can be found in the 'Controls' option.

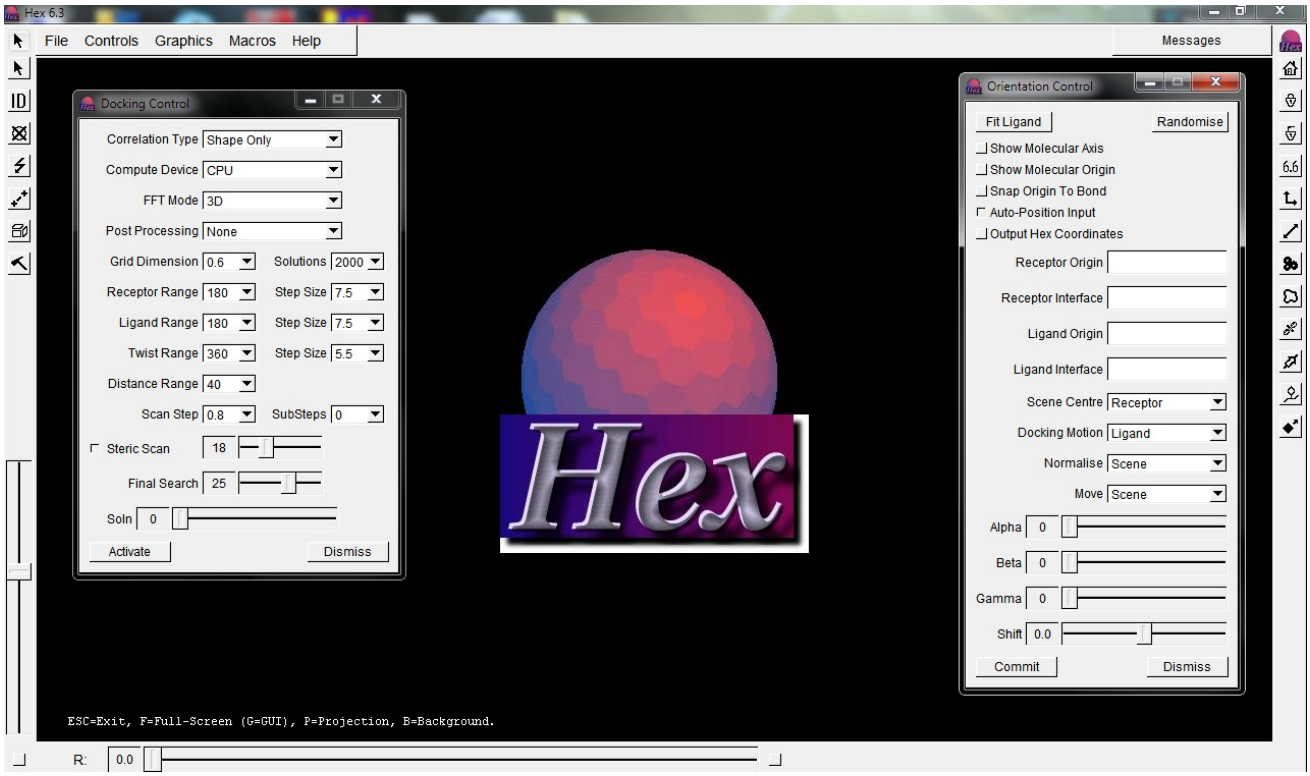


Figure 5.5: Main program window of Hex 6.3 docking program

Once both receptor and ligand proteins are given as input, their structures will appear in the window. The structures can be toggled to different types of view according to user discretion. The docking control is used with following settings:

- Correlation type : Shape only
- Post Processing : Energy minimization
- Solutions: 100
- Scan step: 1.0
- Rest default

Press ‘Activate’ to start the docking process. After the process is completed empirical visualization is done by adjusting the ‘Alpha’, ‘Beta’ and ‘Gamma’ axis using the Orientation Control. ‘Shift’ option is used only to avoid any confusion if we are going to generate an overlapped final image.

Chapter 6

Results and Discussion

Results and Discussion

6.1 Sequence Alignment

The BLASTp of Curcin protein sequence was performed and related sequences based on 50% sequence similarity and minimum E-value was collected. Multiple sequence alignment was done with the help of CLUSTAL X software using default parameters. CLUSTAL X arranges the backbone of the target sequence according to that of the template, using the sequence alignment to decide where to position each residue. The conserved sequence was then generated and used for modeling. The MSA graph is attached in the Appendix of this thesis report.

6.2 Homology Modeling

While modeling the Curcin protein, Momocharin A chain was used as a template. This protein has 33.47 % sequence identity with Curcin, thus homology modeling was done using this template on the SWISS-MODEL web server. Three sets of models were generated by satisfaction of spatial restraints. Following were the data obtained after modeling:

Table 6.1 : SWISS MODEL QMEAN4 scores

Curcin	Raw scores	Z - Score
Model 1	**0.485**	**- 4.03**
Model 2	**0.485**	**- 4.05**
Model 3	**0.485**	**- 4.06**

Out of these three models, Model 1 was selected for further processes based on lowest Z-score.

6.3 Structure Analysis and Verification

The models obtained after homology modeling were analyzed with RAMPAGE (Ramachandran Plot server) for further structure verification. The results obtained are:

Table 6.2: Analysis of Ramachandran Plot for Secondary Structure Prediction	
Model 1:	Number of residues in favored region (~98.0% expected): 634 (94.1%) Number of residues in allowed region (~2.0% expected): 32 (4.7%) Number of residues in outlier region: 8 (1.2%)
Model 2:	Number of residues in favored region (~98.0% expected): 635 (94.2%) Number of residues in allowed region (~2.0% expected): 30 (4.5%) Number of residues in outlier region: 9 (1.3%)
Model 3:	Number of residues in favored region (~98.0% expected): 628 (93.2%) Number of residues in allowed region (~2.0% expected): 32 (4.7%) Number of residues in outlier region: 14 (2.1%)

Model No.1 was selected as the best model as per Ramachandran Plot analysis since it has least number of residues in outlier region.

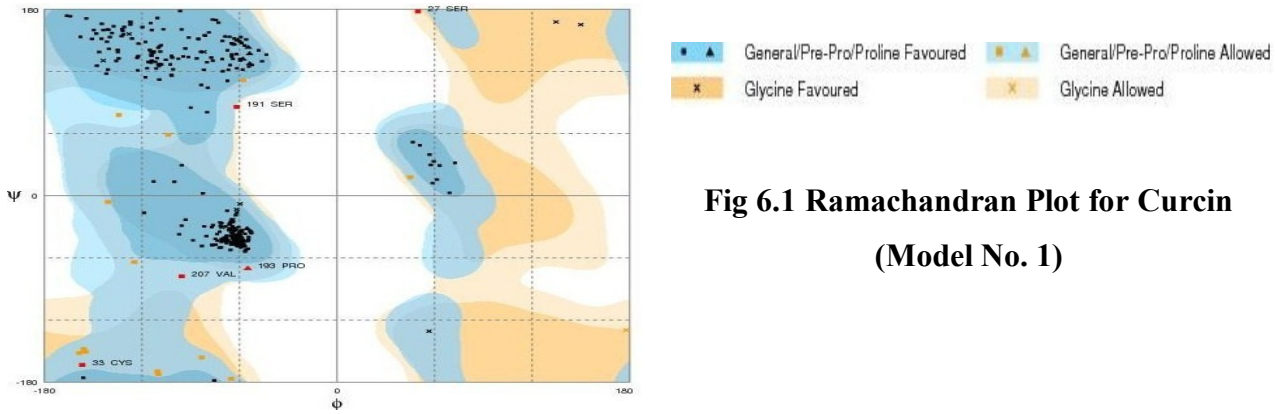


Fig 6.1 Ramachandran Plot for Curcin (Model No. 1)

6.4 Structure minimization and visualization using Chimera and PyMol of proteins under study

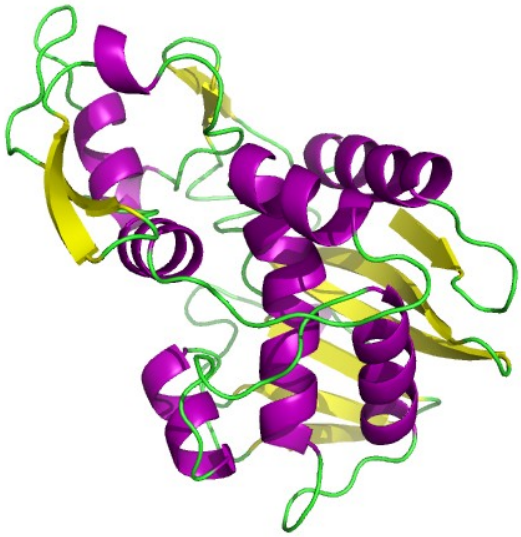


Figure 6.2 (a): Curcin

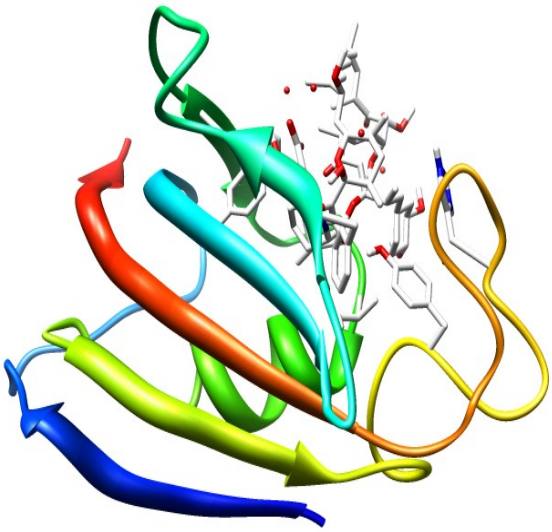


Figure 6.2 (b): mTOR with Rapamycin

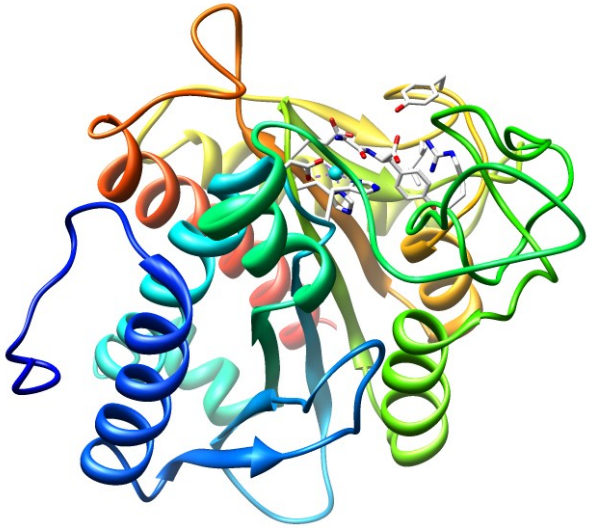


Figure 6.2 (c): Potato Carboxypeptidase Inhibitor

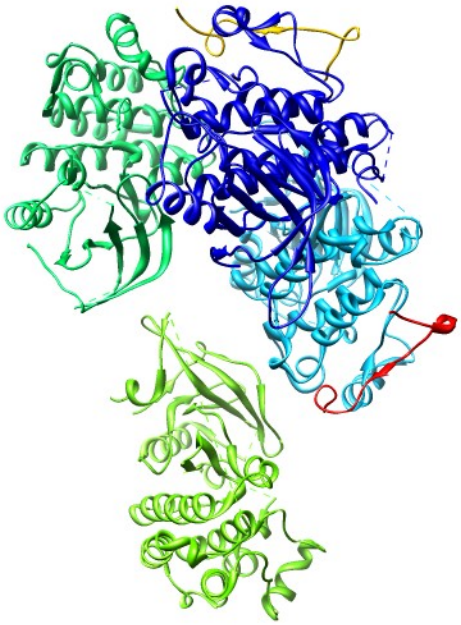


Figure 6.2 (d): EGFR intracelluar domain

6.5 Protein-Protein docking studies

Docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex.

Interaction of Curcin with mTOR

After docking operation of ligand 'Curcin' with 'mTOR' receptor it is observed that the curcin binds preferentially to the opposite side of Rapamycin binding region which is responsible for inhibition of mTOR in the signal transduction pathway.

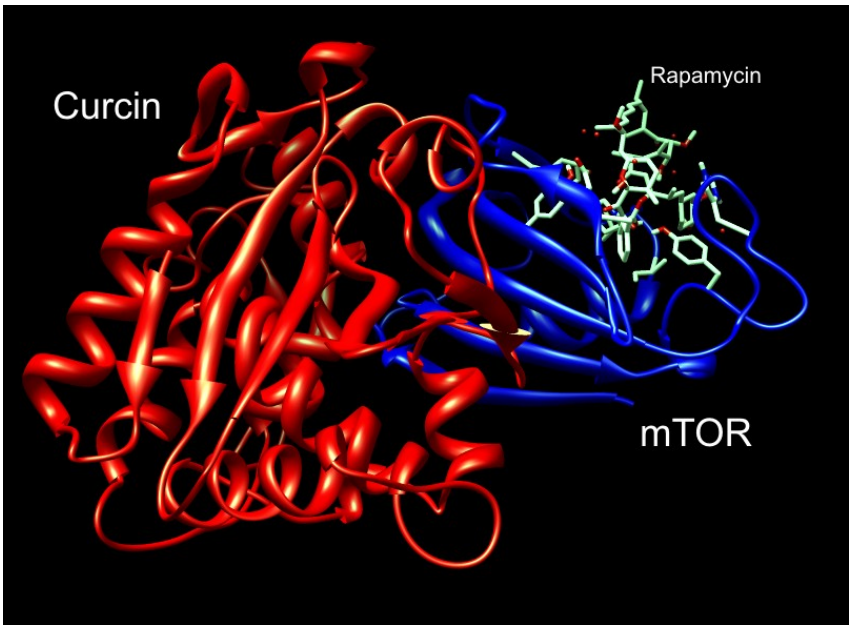


Figure 6.3: Showing binding of Curcin with mTOR in cartoon view

Discussion: The above observation indicates that Curcin does not have a direct role in signaling pathway via mTOR. As mTOR – Rapamycin complex formation is necessary for inhibition of cell proliferation by arresting the cell in G1 phase; the role of Curcin is not significant in this respect. However the limitations of *in-silico* study apply here very well as the above interaction may not be practically feasible *in-vivo*.

Interaction of Curcin with EGFR

The docking operation of 'Curcin' ligand was performed with 'EGFR complex intracellular domain' as receptor. The results were compared with docking operation using 'Potato Carboxypeptidase Inhibitor' ligand as positive control.

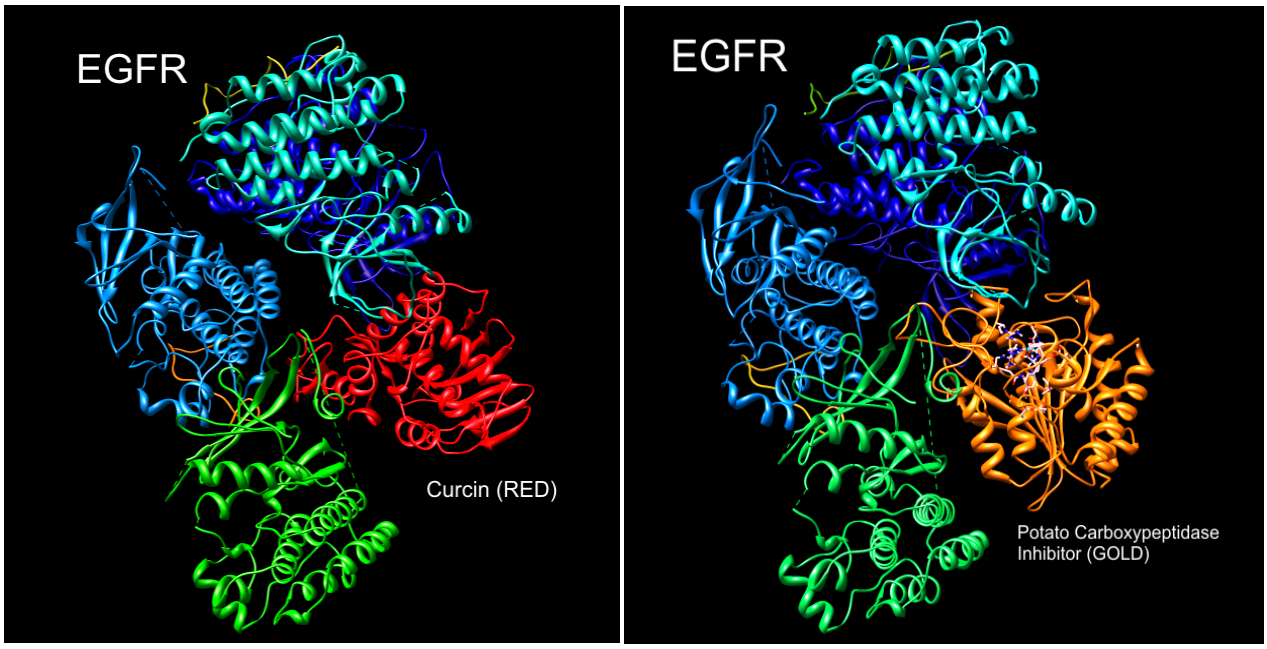


Fig 6.4 (a): Cartoon image showing binding of Curcin[RED] with EGFR (left) and Potato Carboxypeptidase Inhibitor[GOLD] with EGFR (right)

Potato Carboxypeptidase Inhibitor (PCI) being a protease inhibitor is observed to bind with the tyrosine kinase domain of EGFR. This site specific binding of ligand with EGFR inhibits formation of receptor dimer and also prevents receptor trans-autophosphorylation, thus inhibiting cell proliferation. Curcin is also observed to show a similarity in binding properties with EGFR targeting the same domain of EGFR as done by PCI. Further images below show the localization of both proteins in the same EGFR domain as seen by the merged image of individual binding results.

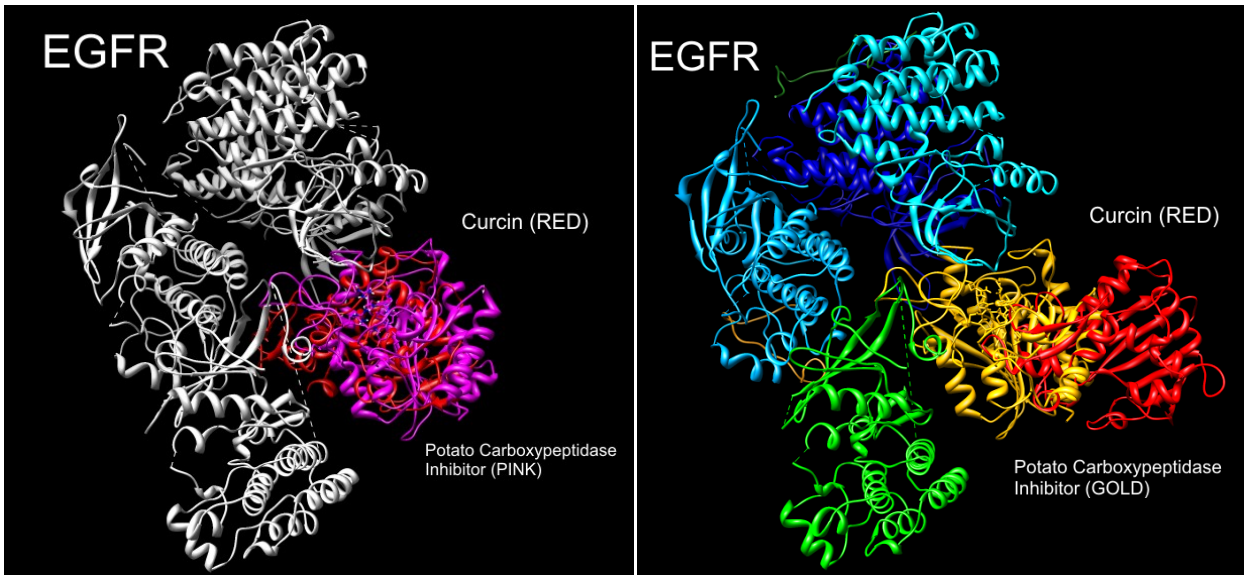


Fig 6.4 (b): Cartoon image showing co-localization of Curcin [RED] and PCI [PINK] in the same domain of EGFR (left). The image is created by merging images of individual docking data. To aid visually the Curcin [RED] is shifted a bit so that PCI [GOLD] is visible clearly (right).

The E-total value obtained from the above two docking procedures were observed as show below:

Table 6.3: Results obtained after performing docking using HEX 6.3

Orientations	Curcin	PCI
1	-266.80	-253.50
2	-262.80	-246.60
3	-225.30	-216.90

Discussion: The comparable E-total values suggest that successful docking of Curcin with EGFR tyrosine kinase domain is energetically possible as in the case of natural inhibitor protein PCI. Therefore it can be predicted that Curcin can also inhibit cell proliferation by preventing the EGFR signal transduction pathway, thus acting as an anti tumor agent.

Chapter 7

Conclusions

Conclusions

By modeling Curcin, we identify useful templates that are showing good similarity with Curcin and show an antitumor activity. Interestingly, one of the models derived from comparative or homology modeling in SWISS-MODEL (best QMEAN4 value) was validated and displayed several meaningful features: secondary structure, charge distribution, conserved residues engaged in non-bonded interaction. Since the above work is an *in-vitro* study, the predicted protein model of Curcin (which proves its efficacy after docking studies) has to go for clinical trials to establish its effectiveness to develop new inhibitor against tumor. The laticifer proteins of *Jatropha curcas* can be further tested on mammalian cell lines both normal and cancerous in future for analyzing the growth potential for use of laticiferous extract as a natural medication. The above work aims to serve all those researchers for further studies to help the patients who are currently experiencing this disease. As of till now only little information is available about the biochemical and pharmaceutical properties of *Jatropha curcas* latex. Hopefully this study will help in aiding further developments in field of natural therapeutic approaches.

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Appendix

CLUSTAL 2.0.12 MULTIPLE SEQUENCE ALIGNMENT

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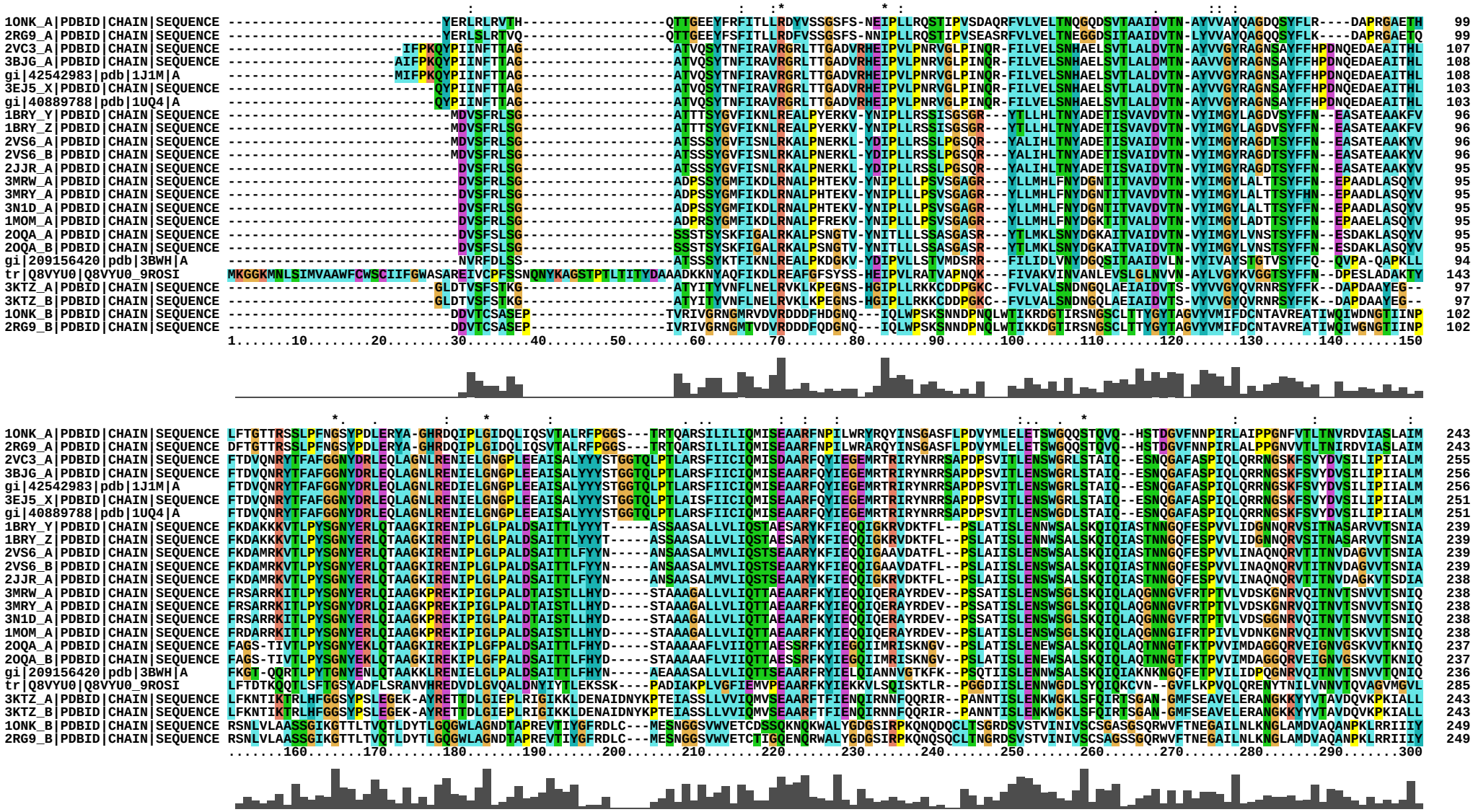
Page 1 of 2



CLUSTAL 2.0.12 MULTIPLE SEQUENCE ALIGNMENT

File: /home/sandeep/Desktop/sequences/Curcin/curcin 293.ps **Date: Wed May 4 16:18:03 2011**


```
1ONK_A|PDBID|CHAIN|SEQUENCE LFVCGERPSSS---   254
2RG9_A|PDBID|CHAIN|SEQUENCE LFVCGE--------   249
2VC3_A|PDBID|CHAIN|SEQUENCE VYRCAPPPSSQF--   267
3BJG_A|PDBID|CHAIN|SEQUENCE VYRCAPPPSSQF--   268
gi|42542983|pdb|1J1M|A      VYRCAPPPSSQF--   268
3EJ5_X|PDBID|CHAIN|SEQUENCE VYRCAP--------   257
gi|40889788|pdb|1UQ4|A      VYRCAPPPSSQF--   263
1BRY_Y|PDBID|CHAIN|SEQUENCE LLLNRNNIA-----   248
1BRY_Z|PDBID|CHAIN|SEQUENCE LLLNRNNIA-----   248
2VS6_A|PDBID|CHAIN|SEQUENCE LLLNRNNMA-----   248
2VS6_B|PDBID|CHAIN|SEQUENCE LLLNRNNMA-----   248
2JJR_A|PDBID|CHAIN|SEQUENCE LLLNRNNMA-----   247
3MRW_A|PDBID|CHAIN|SEQUENCE LLLNTKNI------   246
3MRY_A|PDBID|CHAIN|SEQUENCE LLLNTKNI------   246
3N1D_A|PDBID|CHAIN|SEQUENCE LLLNTKNI------   246
1MOM_A|PDBID|CHAIN|SEQUENCE LLLNTRNI------   246
2OQA_A|PDBID|CHAIN|SEQUENCE LLLN----------   241
2OQA_B|PDBID|CHAIN|SEQUENCE LLLN----------   241
gi|209156420|pdb|3BWH|A     LLLNIGATA-----   245
tr|Q8VYU0|Q8VYU0_9ROSI      LNAVNYKV------   293
3KTZ_A|PDBID|CHAIN|SEQUENCE KFVDKDPK------   251
3KTZ_B|PDBID|CHAIN|SEQUENCE KFVDKDPK------   251
1ONK_B|PDBID|CHAIN|SEQUENCE PATGKPNQMWLPVF   263
2RG9_B|PDBID|CHAIN|SEQUENCE PATGNPNQMWLPVP   263
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